Mouse Skin Is Particularly Susceptible to Tumor Initiation During Early Anagen of the Hair Cycle: Possible Involvement of Hair Follicle Stem Cells

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Stem cells are believed to be a necessary target of chemical carcinogens. Based on autoradiographic, ultrastructural, and biologic criteria, we have recently proposed that hair follicle stem cells reside not in the bulb, but in the upper outer root sheath in an area called the bulge. Proliferating cells have been shown to be more susceptible to tumor initiation, and we have recently demonstrated that cells in the bulge undergo transient proliferation during early anagen. Therefore, we theorized that mouse skin should be particularly susceptible to carcinogen application during early anagen phase. In this paper, we show that early anagen Swiss and Sencar mouse skin is indeed particularly susceptible to one- and two-stage chemical carcinogenesis, resulting in tumor yields one to five times those obtained with telogen-timed carcinogen application. Our findings implicate a possible involvement of the bulge cells as precursors to some of the skin cancers, and support the concept that these are stem cells. These observations also raise important questions about the cellular origins and biologic behavior of chemically induced murine skin tumors. Key words: epithelial stem cells/hair biology/carcinogenesis. *J Invest Dermatol 101:591–594, 1993*

Mouse skin provides an important experimental model system for the study of chemical carcinogenesis [1–3]. In one-stage carcinogenesis, animals are treated with a large dose of a "complete" carcinogen, resulting in the development of skin tumors. In two-stage carcinogenesis, the experiment involves an initial treatment of the skin with a tumor "initiator," which introduces permanent changes in the nuclear DNA of stem cells. Initiation is followed by treatment with a "promotor," which plays a role in tumor progression presumably through the induction of cellular hyperproliferation. It has been known for some time that the hair follicles play an important role in these murine models of skin carcinogenesis [4], and that topical application of a carcinogen at specific phases of the hair cycle results in striking differences in tumor formation [5–7].

The hair cycle is divided into three phases: a period of sustained growth (anagen), an interval of cellular degeneration and rearrangement (catagen), and a period of rest (telogen [8]). To understand better the complexities of this highly regulated cycle, it is essential to understand what governs the growth properties of follicular epithelial cells. Because the hair follicle is a self-renewing system, it is, by definition, governed by stem cells (for a review of stem cells in general, see [9,10]). Based on cell kinetic, ultrastructural, and several biologic criteria, we have provided evidence that hair follicle stem cells are not located in the lower bulb, as previously thought [8,11,12], but rather in the bulge of the upper portion of the follicle [13–15]. This finding led us to develop the "bulge-activation hypothesis," which explains many previously puzzling features of the hair cycle [13–15]. We have recently confirmed an important tenet of this hypothesis. At the beginning of the growing phase (anagen), the normally slow-cycling stem cells of the bulge area were observed to undergo transient proliferation, giving rise to transient amplifying (TA) cells, which subsequently formed the new downgrowth.‡

In mice, the first and second hair cycles are highly synchronized, starting in a wave-like fashion from the head and progressing distally toward the tail [16,17]. This synchrony makes it feasible to evaluate the dependance of carcinogenesis on the phase of the hair cycle. It has been shown, for example, that in one-stage carcinogenesis protocols, the timing of carcinogen application, in relation to the phase of the hair cycle, appears to be important. Borum [6] and Berenblum *et al* [7] reported that a single carcinogen application during the resting phase (telogen) of the hair cycle resulted in a high tumor yield, whereas similar treatment during the growing phase (anagen) of the hair cycle resulted in no tumor formation. Andreasen and Engelbreth-Holm [5] noted a similar trend. In contrast, preliminary results of Berenblum *et al* [7] indicate that skin in anagen is more susceptible than telogen using a two-stage carcinogenesis protocol.

The above noted discrepancy between one- or two-stage carcinogenesis has never been reconciled. Yet almost all workers in the field of chemical carcinogenesis adhere to a protocol of initiating the animals during the resting (telogen) phase of the hair cycle, for

Manuscript received February 10, 1993; accepted for publication May 22, 1993.

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‡ Lavker RM, Fryer E, Margolis-Fryer, Ostad M, Cotsarelis G, Sun TT: Cells in the bulge region of mouse hair follicle undergo transient proliferation during onset of anagen and in response to physical and chemical stimuli. *J Invest Dermatol* 98:581A, 1992.

0022-202X/93/$06.00

both one- and two-stage carcinogenesis experiments [18–23]. This discrepancy needs to be readressed because carcinogenesis protocols have significantly changed since the early work of Berenblum *et al* [7]. For example, i) acetone is routinely employed as the vehicle for carcinogens instead of benzene, ii) promotion is performed using TPA in acetone instead of croton oil in liquid paraffin, and iii) treated mice are usually observed for much longer than 10 weeks. Therefore, we decided to re-examine the relationship between tumor yield and hair cycle in both one- and two-stage carcinogenesis. In this paper, we provide experimental evidence to show that early anagen mouse skin is particularly susceptible to chemical carcinogenesis regardless of whether one uses a one- or two-stage protocol. We suggest that this susceptibility is related to the transient activation of bulge cells to proliferate at the beginning of anagen. Moreover, our finding is consistent with the hypothesis that these cells are stem cells and strongly implicates a role for these bulge cells in the formation of some chemically-induced skin tumors.

MATERIALS AND METHODS

Determination of Stage of Hair Cycle Swiss and Sencar mice were obtained at 21 d of age. Prior studies [5–7,24,25] have shown this to be the approximate time of the first telogen. Two days later, each mouse was anesthetized with a 0.09 ml subcutaneous injection of 7 mg/ml Nembutal, and a sterile transducer containing a 10-digit identifier sequence (Biomedic Corp) was deposited by subcutaneous injection into the lower mid-back region. The dorsum was then dyed with black hair dye (Clairol Nice N'Easy) diluted 2:1 with 4% hydrogen peroxide, and animals were examined daily. The onset of anagen was heralded by a change in skin color from pink to white, followed a day later by the emergence of white "stubs" at the base of black hairs. When the white portion of the hair reached a stable length, this indicated that hair growth had ceased and mice were in telogen. In a preliminary study, two groups of seven mice were sacrificed when anagen and telogen were clinically observed. Strips of skin were taken from the superior, middle, and inferior portions of the back, and examined histologically. In all instances, the histologic examination was consistent with the clinical observation.

Hair Dying and Tumor Induction Regardless of the experimental carcinogenesis protocol all animals were anesthetized as described above, and their backs were clipped prior to receiving carcinogen. No change in any animal's hair cycle was noted in response to hair clipping. The hair-dying technique described above was used in all carcinogenesis experiments to monitor the hair-cycle phase in each animal at the time of carcinogen application, because even though many experimenters estimate the hair-cycle phase based on animal's age, the timing of these phase changes is variable, and is known to differ with the individual animal, its sex, and litter [25]. To control for the possibility that hydrogen peroxide might have weak promoting ability [26], a group of 30 Sencar mice were dyed as described above, and received weekly topical application of 2 μg of phorbol 12-myristate 13-acetate (TPA) in 0.2 ml of acetone to their mid back for 40 weeks, without prior carcinogen application; none of these animals developed tumors.

Experiments were begun only during the first two hair cycles, because they have been shown to be highly synchronized. The third cycle is less ordered, with hair growth occurring in patches randomly distributed over the body, rather than in a wave-like fashion [7,14,24,25,27,28]. We also noted this asynchrony in 10 of 10 animals followed into a third anagen with a second hair dying (unpublished data). Because of this "breakdown" in the regular wave-like hair growth during third anagen, studies involving the application of carcinogen to animals older than 10–12 weeks (the onset of third anagen), will result in simultaneous initiation of both anagen and telogen skin within the same mouse. Such studies, although providing information about the relationship between aging and cancer development [22,29,30], yield no insight into hair cycle–related effects.

One-Stage Carcinogenesis Protocol One-stage carcinogenesis involved a single topical application of 3000 nmole of dimethylbenzeanthracene (DMBA; Sigma Chemicals), freshly made in acetone, to the mid backs of 30 female Swiss or Sencar mice. The animals were observed weekly for 40 weeks, and the number of tumors (> 1 mm diameter) were recorded for each animal.

Two-Stage Topical Carcinogenesis Protocol Two-stage carcinogenesis protocols entailed initiation by topical application of 30 nmoles of DMBA in acetone, followed 2 weeks later and weekly thereafter for 20 weeks by topical promotion with 2 μg of phorbol 12-myristate 13-acetate (TPA; Sigma Chemical) in 0.2 ml of acetone.

Systemic Initiation Protocols One-stage systemic carcinogenesis involved a single intragastric dose of 3000 nmoles of DMBA in 0.3cc olive oil. In a 2-stage protocol, animals were initiated intragastrically with 3000 nmoles of DMBA, followed 2 weeks later by weekly topical promotion with TPA as described above. In a preliminary study, ten mice were given intragastric applications of 1000–5000 nmols of DMBA in 0.3 cc of olive oil and followed for 4 weeks. At the end of 4 weeks, animals showed no clinical signs of toxicity and no histologic evidence of adrenal toxicity [31,32].

RESULTS AND DISCUSSION

Because stem cells are known to be involved in skin tumor initiation [22,33–35], and because proliferating cells are more susceptible to tumor initiation [36–40], the transient proliferation of putative follicular stem cells suggests that mouse skin should be particularly susceptible to tumor initiation during this time period. However, this is contrary to the conclusion drawn by Berenblum *et al* [7] who found that in one-stage carcinogenesis protocols, Swiss mice treated during telogen with a single high dose of DMBA gave rise to more tumors (0.5/mouse at 10 weeks) than mice treated during anagen (0/mouse). A similar telogen-requirement was reported by Borum [6].

We treated Swiss mice according to a one-stage protocol consisting of a single topical application of 3000 nmole of DMBA during either early anagen or telogen. Ten weeks after the application, the telogen-treated animals yielded 0.5 tumors/animal, whereas the anagen-treated animals showed practically no tumors (Fig 1*a*). This result seemed to confirm the original investigations of Berenblum *et al* [7] and Borum ([6], which were limited to 10 weeks). However, upon continuing the experiment for 25–35 weeks, we found anagen-treated animals actually yielded slightly more tumors than the telogen-treated ones (Fig 1*a*). These results were interesting, but the overall tumor yields were so low in the Swiss mice that the differences were statistically insignificant. Therefore, we repeated these experiments using Sencar mice (Fig 1*c*), which were developed specifically for their greatly enhanced susceptibility to skin chemical carcinogenesis [39,41,42]. The results were similar but much clearer than those of the Swiss mice. Again we found that telogen-treated animals yielded slightly more tumors than the anagen ones up to 20 weeks post-treatment. However, subsequently the tumor yield of the anagen-treated animals rose sharply so that by 40 weeks the anagen values (six tumors/animal) were much greater than telogen (two tumors/animal, Fig 1*c*). This difference was statistically highly significant ($p < 0.001$), and showed that, at least in one-stage carcinogenesis, anagen skin is actually much more susceptible to tumor initiation — contrary to the current belief.

Because two-stage carcinogenesis can dissect different steps of initiation and promotion, with a high tumor yield [1,2], we felt it important to repeat our experiment using this protocol. We again studied both Swiss and Sencar mice. In Swiss mice (Fig 1*b*), the tumor yield of the two-stage protocol (2.5 tumors/mouse at 30 weeks) was five times greater than that of the one-stage protocol (0.5 tumors/mouse, Fig 1*a*). However, in Sencar mice the two-stage protocol (Fig 1*d*) did not further increase the tumor yield over the one-stage protocol (Fig 1*c*). In both types of mice, more tumors were produced when initiation was done in early anagen instead of telogen. This result is consistent with the preliminary two-stage investigations of Berenblum *et al* [7], and again contradicts the current belief of telogen superiority.

One possible cause for the hair cycle–dependent changes in tumor formation is a change in the degree to which a carcinogen can penetrate through the hair canal reaching, for instance, the bulge area. To circumvent the follicular barrier (as well as sebum flow) we initiated a group of Sencar mice systemically. The results of a one-stage protocol (Fig 1*e*) indicate that anagen- and telogen-initiated animals developed comparable numbers of tumors at 35–40 weeks (1–2 tumors/animal). However, results from a two-stage systemic protocol (systemic initiation plus topical TPA promotion, Fig 1*f*) indicate that anagen-initiated animals gave rise to many more tumors (five/animal) than the telogen-initiated animals (two/ani-

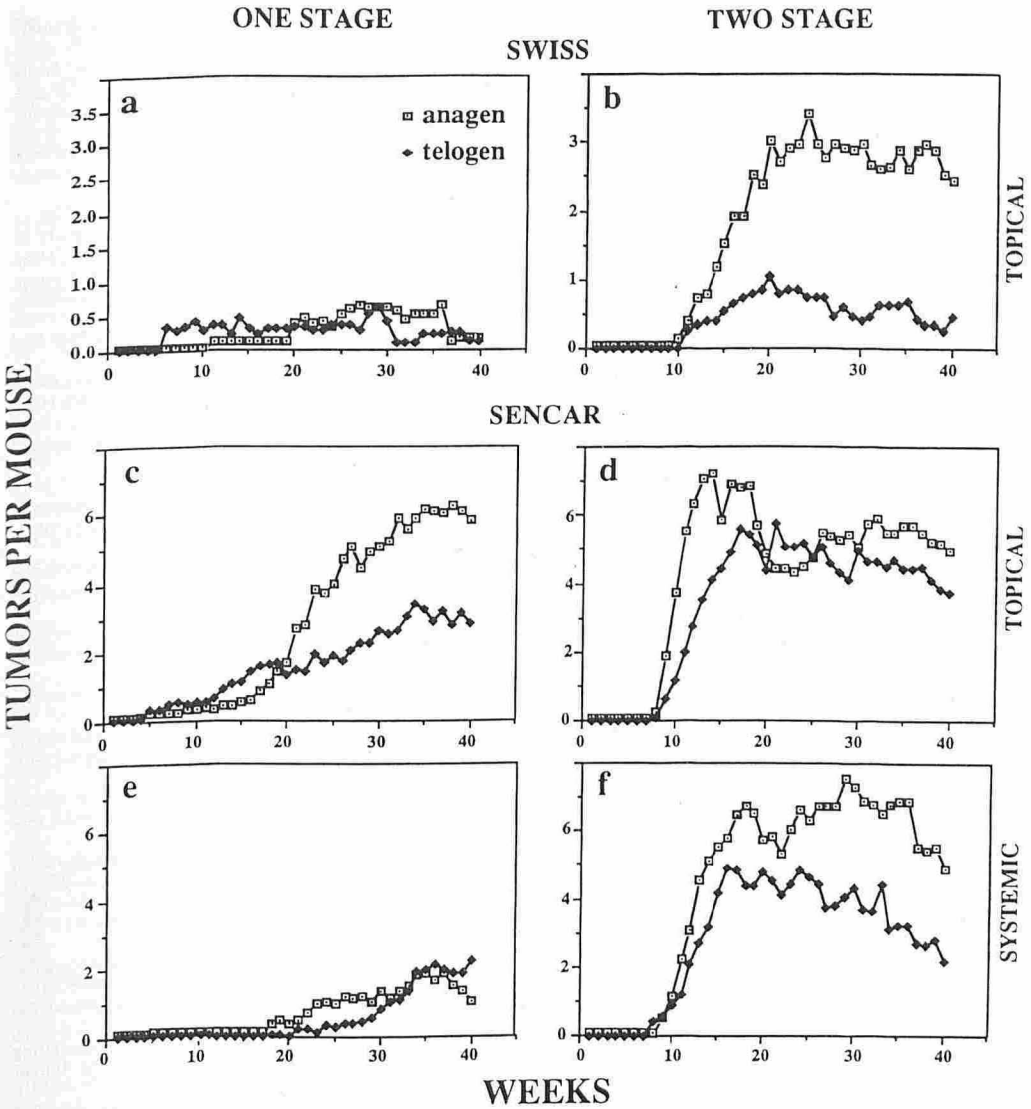

Figure 1. Tumor development in Swiss (*a,b*) and Sencar mice (*c–f*) as a function of whether tumor initiation was done during the anagen versus telogen phase of the hair cycle. Swiss mice received either (*a*) a single topical application of 3000 nmole of DMBA during the onset of anagen or during telogen in a one-stage protocol, or (*b*) a single topical application of 30 nmoles of DMBA followed by weekly TPA promotion in a two-stage protocol. Similar experiments were repeated using Sencar mice using (*c*) one- and (*d*) two-stage protocols. In addition, Sencar mice received either (*e*) a single dose of 3000 nmoles of DMBA intragastrically, in a one-stage protocol, or (*f*) 3000 nmoles of DMBA intragastrically, followed by weekly topical TPA promotion in a two-stage protocol. Tumor initiation was done during anagen or telogen, as indicated. Almost all tumors that developed were papillomas; only a few squamous cell carcinomas occurred that showed no predilection for any experimental group (data not shown). Note that the anagen-initiated animals had significantly greater tumor yields than telogen-initiated animals (two-tailed t test, $p < 0.001$).

mal). Consistent with this finding is our recent observation that when benz(o)pyrene is applied topically to the dorsal skin of Swiss and Sencar mice in early anagen or telogen, no difference in the distribution or persistence of benz(o)pyrene is seen over a 7-d period regardless of the stage of the hair cycle.§ This result is consistent with that of two-stage topical experiments. It proves conclusively that follicular barrier and/or sebum flow do not play a significant role in shaping our results, and that anagen skin is intrinsically more susceptible to chemical carcinogenesis.

Therefore, all our results are consistent with and support the concept that tumor yield can be significantly affected by the hair cycle [24,43–46]. Moreover, our data clearly indicate that, contrary to common belief, anagen initiation gives rise to significantly greater numbers of tumors than telogen initiation. Interestingly, this time period of greater tumor susceptibility coincides with the transient proliferation of bulge cells. This correlation is consonant with the hypothesis that these cells are stem cells and implicates such cells as possible precursors to some of the skin cancers. The possible involvement of bulge/follicular stem cells in skin chemical carcinogenesis closely parallels the situation in the cornea. In this case the limbus is thought to be the exclusive site of corneal epithelial stem cells [47], and also happens to be the predominant site of origin of corneal squamous cell carcinomas [48].

An important corollary of the bulge/follicular involvement in tumorigenesis is that it should not be assumed that all chemically induced mouse skin cancers are derived from the interfollicular epidermis, as is implicitly assumed in many published studies [18–23,36–39,41,42]. This means that the relationship among the different populations of skin keratinocytes and skin tumors needs to be studied in the future. This question is of particular interest in view of the proposal that the follicular bulge cells may represent a population of pluripotent stem cells that can give rise to not only the hair follicle but also the epidermis and sebaceous glands [13–15]; cf [49,50]. Other important issues that need to be further addressed in the future include the following. i) Is the cellular origin (e.g., hair follicle versus interfollicular epidermis) of anagen-initiated tumors similar to telogen-initiated tumors? ii) Are anagen-initiated tumors histologically similar to telogen-initiated tumors? iii) Do anagen-initiated tumors behave in the same way as telogen-initiated tumors with respect to progression from benign papilloma to squamous cell carcinoma? Answers to these questions may yield useful insights

§ Wilson CL, Sun T-T, Lavker RM: Hair cycle has no effect on distribution and persistence of a topically applied carcinogen in mouse skin. *J Invest Dermatol* 98:658A, 1992.

into the behavior, progression, and physiologic relevance of some of the experimentally induced tumors of mouse skin.

We thank S.H. Yuspa and H. Hennings of the National Cancer Institute for valuable discussions and helpful suggestions.

This work was supported by National Institutes of Health Grants AR39674 and EY06769 (RML), AR34511, AR39749, and EY4722 (T-TS), AR41669 (RML and T-TS), the National Alopecia Areata Foundation (RML and T-TS), and the Medical Research Council, United Kingdom (CW).

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